LavaLAMP™ RNA Master Mix

Please read carefully and thoroughly before beginning

FOR RESEARCH USE ONLY. NOT FOR HUMAN OR DIAGNOSTIC USE.

IMPORTANT!
-20 °C Storage Required
Immediately Upon Receipt

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Technical Support

Lucigen is dedicated to the success and satisfaction of our customers. Our products are tested to assure they perform as specified when used according to our recommendations. It is imperative that the user-supplied reagents are of high quality. Please follow the instructions carefully and contact our technical service representatives if additional information is necessary. We encourage you to contact us with your comments regarding the performance of our products in your applications. Thank you.

Lucigen Technical Support:
Email: techsupport@lucigen.com
Phone: (888) 575-9695

Product Guarantee: Lucigen guarantees that this product will perform as specified for one year from the date of shipment. Please avoid using reagents for greater than one year from date of receipt.

Product Description

The LavaLAMP™ RNA Master Mix is intended to simplify development and optimization of loop-mediated isothermal amplification (LAMP) reactions. LAMP kits are commonly available as multi-component kits that require optimization (e.g., $MgSO_4$, betaine, enzyme; as well as temperature, primer concentration, etc.). The LavaLAMP™ RNA Master Mix greatly simplifies reaction optimization by limiting optimization to

target-specific components and conditions (LAMP primers, target concentration, and reaction temperature). This kit is for research purposes only, under the limited-use license described at the end of this document.

LAMP commonly employs a set of six primers (F3, B3, Loop-F, Loop-B, FIP, and BIP), which must be supplied by the user. Previously-established primer designs may be used. Alternatively, Lucigen recommends use of the free Eiken web utility or the commercially available LAMP Design Software from Premier BioSoft to design new primer sets (see Appendix A). However, not all primer sets identified by these programs are guaranteed to work with LavaLAMP™ RNA Component Kit or any other LAMP system. We strongly encourage designing multiple primer sets to identify the best performing set. We also highly recommend inclusion of loop primers (Loop-F and Loop-B; Nagamine, 2002) to improve assay performance.

LavaLAMP™ RNA amplification products may be detected by agarose gel electrophoresis or by real-time or end-point monitoring with fluorescent double-stranded DNA-binding dyes, such as the Lucigen Green Fluorescent Dye (Cat. No. 30078-1). Turbidity may also be monitored to assess amplification (Mori, 2001), but this method is less sensitive.

Product Designations and Kit Components

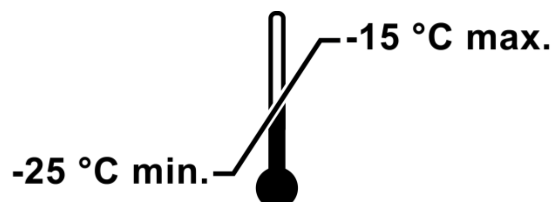
Product	Kit Size	Catalog Number	Reagent Description	Part Numbers	Volume
LavaLAMP™ RNA Master Mix	200 Reactions	30086-1	LavaLAMP™ RNA Master Mix	F824234-1	2 x 1.25 mL
			RNA Positive Control LAMP Primer Mix	F814233-1	25 µL
			RNA Positive Control	F824232-1	10 µL

Product	Kit Size	Catalog Number	Reagent Description	Part Numbers	Volume
LavaLAMP™ RNA Master Mix with Dye	200 Reactions	30087-1	LavaLAMP™ RNA Master Mix	F824234-1	2 x 1.25 mL
			RNA Positive Control LAMP Primer Mix	F814233-1	25 µL
			RNA Positive Control	F824232-1	10 µL
			Green Fluorescent Dye	F883827-1	200 µL

Product	Kit Size	Catalog Number	Reagent Description	Part Numbers	Volume
Green Fluorescent Dye	200 Reactions	30078-2	Green Fluorescent Dye	F883827-1	200 µL

Components and Storage

Store all kits and components at -20 °C



Materials Supplied by the User

- 10X target-specific LAMP primer mix
    - 2 µM each, F3 and B3 primers
    - 8 µM each, Loop-F and Loop-B primers
    - 16 µM each, FIP and BIP primers

      ***Note**: See LAMP Reaction Optimization section for additional details on primers*
- Target RNA
- Thermocycler or heat block

  ***Note:** We recommend using calibrated instruments with heated lids.*
- **Optional**: Use of the Green Fluorescent Dye (Cat. No. 30078-2) for detection of amplified DNA requires measurement of fluorescence at 520 nm with a real-time amplification instrument or a fluorometer for end-point analysis.

  Lucigen has used the following instruments to successfully detect amplified product by fluorescence in combination with the Green Fluorescent Dye: AmpliFire (Douglas Scientific), CFX96 and iQ5 Thermocyclers (Bio-Rad), ESEQuant TS2 (Qiagen), Genie II (OptiGne), ABI 7500 Real-Time PCR System (Thermo Fisher Scientific).

  Fluorescent dyes such as EvaGreen, SYTO-13, and V13-01184 have also been used successfully with the LavaLAMP™ RNA Master Mix, but optimization of dye concentration is necessary to produce the fastest times to results.

Before You Start:

1. For new targets, please refer to the **LAMP Reaction Optimization** section (p.6) to design primer sets and test reaction temperatures.
2. Always wear gloves while handling components. Set up reactions using good laboratory techniques that minimize cross contamination.
3. Thaw and hold reagents on ice and set up reactions on ice to avoid background amplification.
4. Calculate the total volume of each reagent required for the planned experiment and verify that enough reagent is available before proceeding to reaction setup.
5. Decide on the method you will use to detect amplified products. Here are the three most common methods:
    a. **Fluorescent Assays in Real-Time Detection Instruments:** Monitor reaction fluorescence using the FAM or SYBR Green channel to detect amplified product.
    b. **End-point Fluorescent Assays**: Measure fluorescence in a fluorometer using the FAM or SYBR Green channel to detect amplified product.
    c. **Non-Fluorescent End-point Assays**: Agarose gel (visual), spectrophotometer (turbidity, OD_{600})
6. Set a thermocycler or heat block to the desired temperature. If using a heat block, we recommended using 0.2 mL PCR tubes, and monitoring the temperature closely.
7. Lucigen strongly encourages all users to perform a No Target Control (NTC) reaction and new users to also include a Positive Control reaction to aid in troubleshooting.

Reaction Setup

The LavaLAMP™ RNA Master Mix contains all components required for amplification, including Magnesium Sulfate ($MgSO_4$). If you include the Green Fluorescent Dye in the reactions, you can detect the amplified products using a real-time instrument or fluorometer. Alternatively, you can detect amplified products by agarose gel electrophoresis or turbidity using a spectrophotometer.

1. Thaw all kit components on ice and keep them on ice as you set up the reactions.
2. **Mix each component thoroughly before use by vortexing for three to ten seconds.** Centrifuge briefly to collect contents.
3. Prepare initial reaction mix(s) in a single tube in the order listed below in (Table 1). Keep the reaction mixes and all reaction tubes or plates on ice to reduce non-specific background amplification. The No Target Control (NTC) reaction using the Target-specific primer set(s) is strongly recommended to demonstrate a lack of background amplification within the reaction time(s) tested.

   Notes:
   - ***LavaLAMP™ RNA Master Mix cannot be used with PCR or Bst DNA Polymerase reaction conditions.***
   - ***Add Green Fluorescent Dye fresh when preparing reactions, do not store the combined dye and master mix.***
   - *The recommended reaction conditions (Table 1) are for use with Green Fluorescent Dye and 1 µL of Target RNA Sample. Adjust the volume of nuclease-free H_2O when using other amounts of dye or target RNA sample.*
   - *Table 1 provides volumes for a single reaction. If multiple reactions are required, increase volumes proportionately. Prepare enough reaction mix cocktail(s) for the number of amplification reactions being performed plus an additional 10% to accommodate slight pipetting errors.*

Table 1. Recommended Setup of Control and Experimental (Default) Reactions

	Positive Control	**No Target Control (NTC)**	**Experimental**
Component	**Amount (µL)**	**Amount (µL)**	**Amount (µL)**
Nuclease-free H_2O	8	8	8
LavaLAMP™ RNA Master Mix (2X)	12.5	12.5	12.5
Target-Specific Primer Mix, 10X	--	2.5	2.5
RNA Positive Control LAMP Primer Mix	2.5	--	--
Green Fluorescent Dye **(optional)**	1	1	1
Total Volume	24	24	24

4. After all reagents have been added, mix the reaction completely by pipetting several times.
5. If more than one reaction is being run, dispense 24 µL of the reaction mix(es) for each reaction into PCR tubes or a 96-well PCR plate.

   ***Note**: To minimize cross-contamination, perform steps 6 - 8 in an area separate from that used to prepare the reaction mix.*
6. Add 1 µL of Target RNA or Positive Control RNA to the appropriate reaction tubes or wells. Add 1 µL of nuclease-free water to the NTC reaction tubes or wells. Mix completely by pipetting.
7. Cap tubes or seal plate wells. Centrifuge briefly to collect contents prior to incubation.
8. Depending on the detection method chosen, incubate the reactions as follows in a real-time detection instrument, thermocycler, or heat block:

Step	Temperature	Time
1. Amplification	**Experimental and NTC**: 68˚C – 74˚C **Positive Control**: 68˚C	30 - 60 minutes
2. Hold (Optional)	4˚C	∞

9. Detect amplified products using your method of choice.
    a. **For fluorescent assays in real-time detection instruments**: Monitor fluorescence using the FAM or SYBR Green channels at 15-30 second intervals for 30-60 minutes.
    b. **For fluorescent or non-fluorescent end-point assays**: Immediately stop enzyme activity by one of the following methods and then assay using your detection method of choice:
        1. Place on ice or at 4°C.
        2. Add gel loading dye that yields a final concentration of 10 mM EDTA.
        3. Heat-kill the reaction at 95°C for 5 minutes in a thermocycler or heat block.

Note: *Amplified reactions may be kept at -20°C for long-term storage.*

LAMP Reaction Optimization

Target-specific LAMP Primer Mix Design, Quality, and Concentration

Lucigen strongly recommends designing and testing multiple LAMP primer sets, because primer design is extremely important in optimizing LAMP results. For details on primer design, please see Appendix A and Appendix B.

Lucigen recommends HPLC purification for the FIP and BIP primers. Standard desalting may be used for the Loop-F, Loop-B, F3, and B3 primers, but HPLC purification of all primers produces optimal results. Resuspend primers in nuclease-free water or low TE (10 mM Tris-Cl, 0.1 mM EDTA, pH 8.0).

The recommended 10X Target-specific LAMP Primer Mix is:

- 2 µM each F3 and B3 primers
- 8 µM each Loop-F and Loop-B primers
- 16 µM each FIP and BIP primers

Target RNA

Lucigen recommends using purified target RNA for development of a new LAMP assay prior to testing different sample types (e.g., fluids, tissue, environmental). The minimum amount of required target RNA is assay specific. We recommend using at least $1x10^3$ copies per reaction.

Instrumentation

We recommend the use of real-time detection instrumentation for optimization of the LAMP reaction. Real-time instrumentation enables more precise monitoring of the resolution between the positive reactions and potential background amplification. If real-time instrumentation is not available, please refer to Appendix C for the use of end-point detection for optimization.

Primer Set Selection and Temperature Optimization

Individual primer sets have optimal reaction temperatures. The combination of primer set and temperature have significant impact on the speed of the reaction and background amplification. A temperature optimization is recommended on new designs or existing designs that have never been run with LavaLAMP™ RNA Master Mix. The suggested range of reaction temperatures to test with each primer set is 68°C – 74°C.

Recommendations

- Set target input at a moderate level, e.g., $1 X 10^3$ per reaction.
- Screen at least three different primer designs at the default 1X reaction conditions (Table 1, p.5).
- Run each primer set in replicate across the reaction temperature range of 68°C – 74°C
- Run a known positive sample and NTC for each primer set

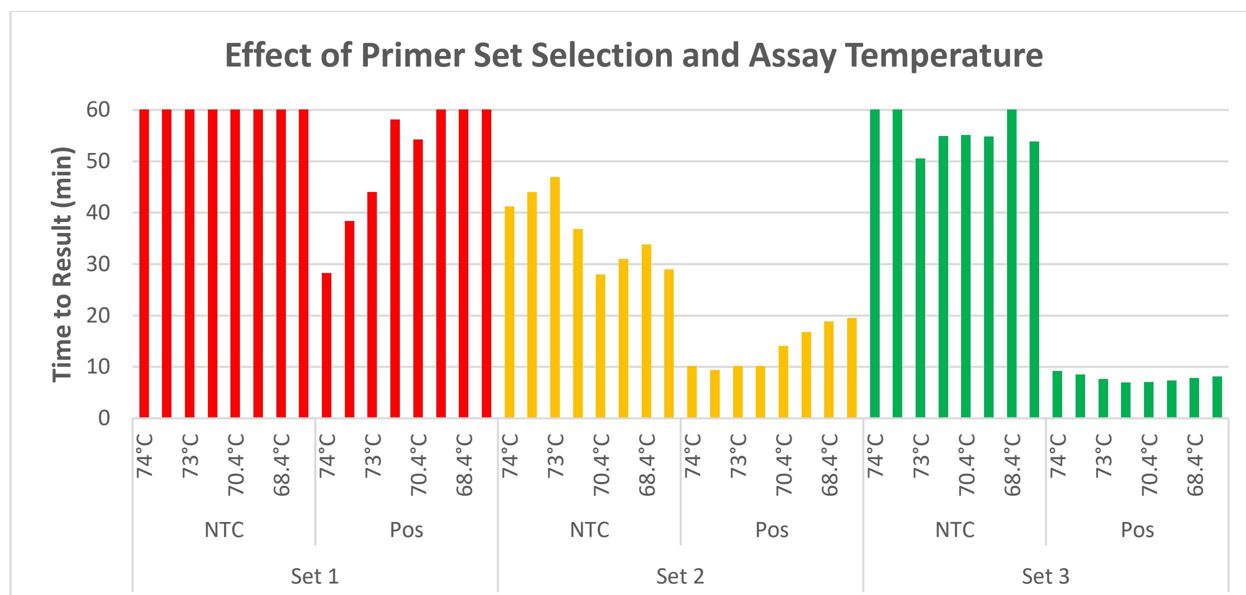


Figure 1. Effect of primer set and temperature on LAMP results. Three primer sets were screened across a temperature range of 68°C – 74°C with recommended 1X default reaction conditions. Real-time detection was performed on a CFX96 Thermal Cycler (Bio-Rad) using Green Fluorescent Dye (Lucigen). Primer Set 3 provided the fastest positive Time to Result with the least amount of background amplification. Within Primer Set 3, 74°C provided the best resolution between the positive and negative samples. Note: Exact temperatures were set by the CFX96 Thermal Cycler instrument within the selected temperature range. Specific temperatures tested will depend on the real-time instrument used.

Primer Concentration Titration (optional)

Depending on the primer-template system, the primer concentration may need to be optimized after the best reaction temperature is identified. Certain primer sets may be prone to background amplification at or near the commonly used LAMP primer concentrations. If undesired background amplification is observed, the primer concentration may be titrated from 0.25X – 1X. The concentration of all primers may be adjusted in unison by adding varying amounts of the 10X Target-specific LAMP Primer Mix. Reducing the primer concentration may reduce sensitivity and reaction yield, or it may increase the time required to amplify your target. Lucigen does not recommend increasing primer concentration above the recommended levels because doing so generally leads to increased background amplification.

Typical LAMP Results

Agarose Gel Analysis of LAMP Reaction Products

LavaLAMP™ RNA amplification products may be analyzed by agarose gel electrophoresis to verify the specificity of the reaction. A positive LAMP reaction should generate a ladder of products, typically consisting of 1-10 visible concatemers of the reaction target. In contrast, non-specific amplification typically generates an undefined smear of DNA, with no distinct banding pattern (see Figures 2 and 3).

Correct Target-specific Amplification

1 2 3 4

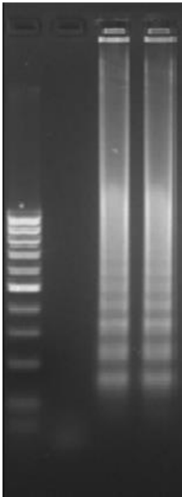
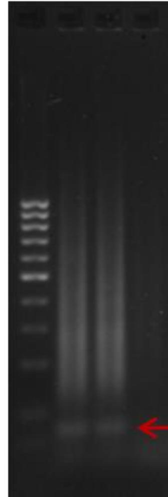


Figure 2: LAMP Reaction Products from a Positive Control Target. Lane 1: 100 bp Marker, Lane 2: No Target Control reaction. Lanes 3 and 4: A distinct banding pattern is seen among the smear, which is indicative of a positive LAMP reaction.

Spurious Background Amplification

1 2 3 4



Primer dimers

Figure 3: Typical Background Amplification in a LAMP Reaction. Lane 1: 100 bp ladder, Lanes 2 and 3: Non-specific or background amplification appears as a smear of DNA fragments with no visible or distinct bands. A prominent primer dimer band is also characteristic of non-specific amplification. Lane 4: Absence of non-specific amplification (no products).

Fluorescent Signals from Different LAMP Reactions

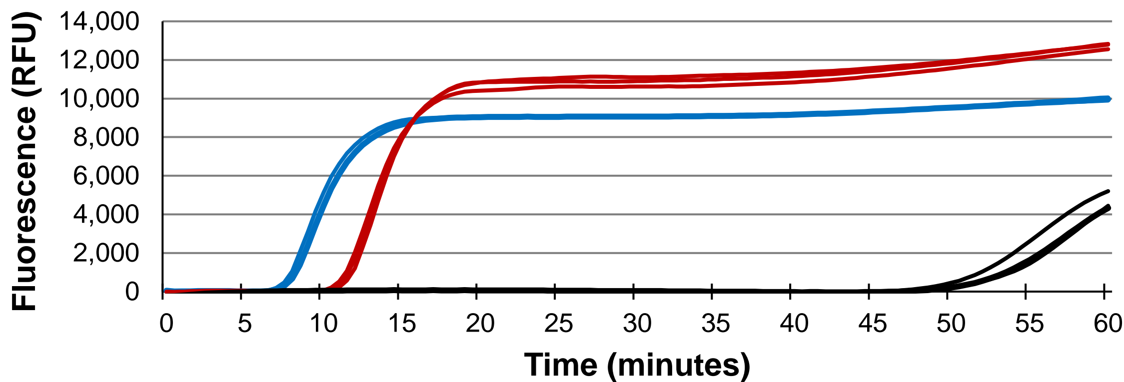


Figure 4: Early Fluorescent Signals from Positive LAMP Reactions and Late Background Signals from NTC Controls after Extended Incubation. LAMP reactions were run in a real-time thermal cycler. The fluorescent signal from each reaction was captured over a 60 minute reaction time. The red and blue lines represent the fluorescent signals from Positive Control reactions with varying amounts of target. The black lines indicate non-specific background amplification signals that can arise later in a No Target Control reaction.

Additional Amplification Guidelines

Prevent Target RNA Contamination

LAMP reactions are very sensitive to contamination by target RNA or amplicon carryover, which can result in false positive amplification. To prevent contamination of LAMP reactions with target RNA or target amplicons, designate and use a specific laboratory area for reaction setup that has never been exposed to the target RNA or amplified products. Use a second area that has never been exposed to amplified material to add your target RNA to your reactions. Finally, designate a third area to analyze LAMP reaction products.

Cold Reaction Setup

The LavaLAMP™ RNA Master Mix exhibits residual activity above 4°C, which can cause non-specific background amplification in the subsequent reaction. The following steps can minimize this source of background

- All LavaLAMP™ RNA Master Mix reactions should be set up on ice and maintained at 4°C prior to amplification.
- Primers should be added just prior to target addition and incubation.
- To start amplification, directly transfer the reactions from ice to a pre-heated heat block or thermal cycler at the correct reaction temperature.

Target Preparation

Most routine methods of RNA target purification are sufficient (e.g. phenol/chloroform or guanidine/silica-based methods), and LAMP reactions generally tolerate some contaminants in the RNA sample. However, trace amounts of purification reagents (e.g. phenol, Proteinase K, ethanol, etc.) may inhibit amplification; therefore, addition of large amounts of sample should be avoided. In addition, EDTA can inhibit amplification, so it is preferable to dissolve the RNA target in water or EDTA-free buffer. If TE must be used, we recommend using low TE (10mM Tris, 0.1 mM EDTA.)

LAMP Reaction Timing

The amplification threshold is usually reached in 8-20 minutes. Thus, 30 minutes is the recommended incubation time for end-point reactions. Longer incubation times may lead to the appearance of undesired background (Figure 3).

Reaction Overlay

A thermal cycler with a heated lid is required to prevent evaporation of the reaction mix. If such an instrument is not available, the reaction mixture can be overlaid with one-half reaction volume of PCR-grade mineral oil. However, mineral oil may slow the reaction.

Appendix

A: Primer Design Software

Lucigen recommends designing multiple LAMP primer sets and testing them to identify the best performing set. The following software is available to help design primer sets:

The Eiken PrimerExplorer is a free online application that can be accessed at: https://primerexplorer.jp/e/

The LAMP Designer by Premier Biosoft, is available for purchase at: http://www.premierbiosoft.com/isothermal/lamp.html

B: Melting Temperature (Tm) Setpoints

LAMP primer design software (including Eiken PrimerExplorer and LAMP Designer by Premier Biosoft) were optimized for Bst polymerase, which has an optimal temperature of 63°C - 65°C. Because the LavaLAMP™ Enzyme has an optimal temperature range of 68°C - 74°C, it is necessary to increase the default Tm setpoints in the software as follows:

	Default Tm Settings	Suggested Tm Settings for LavaLAMP™ RNA
FIP and BIP	65˚C	71˚C
F3 and B3	60˚C	66˚C
Loop-F and Loop-B	62˚C	68˚C

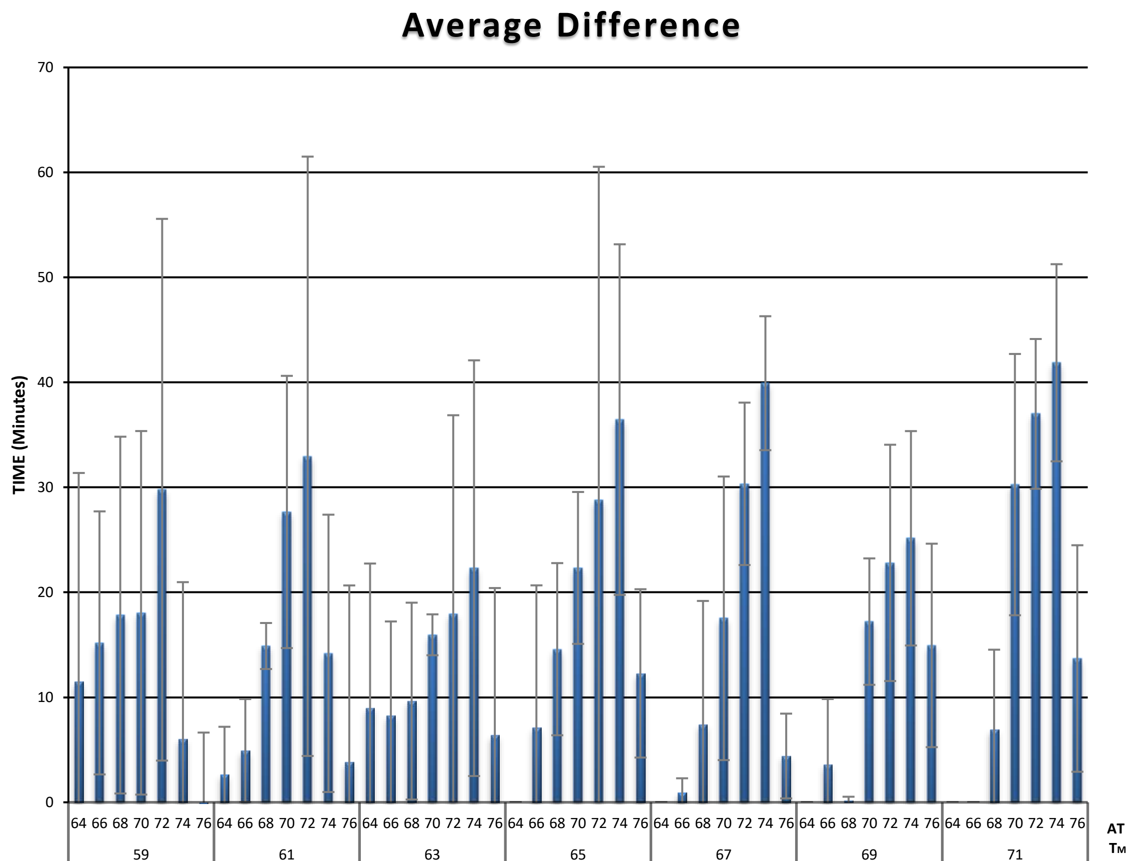


Figure 5: Effect of Tm setpoints in primer design software. The difference between the Time to Result of negative and positive samples for various assay temperatures (AT) for individual reactions and primer Tm setpoints ("TM") for FIP and BIP. In this example, the 71°C setpoint provided the largest difference between positive and negative and the largest operating window for the assay temperature (70°C - 74°C).

C: End-Point Optimization

The preferred method of detection for developing and optimizing LAMP assays is real-time fluorescence with a DNA binding dye; however, it is also possible to use endpoint analysis. Endpoint LAMP assay optimization requires product measurement at three different time points to follow the amount of product generated versus time. Both Positive Target and No Target Control LAMP reactions must be run to compare positive signal versus background signal over time. Once the LAMP product starts to increase over background, it typically reaches maximum product (saturation) within 10 minutes. Measuring the LAMP product at 10, 20, and 30 minutes will indicate whether the LAMP product is below detection, between minimum detection and saturation, or at maximum (saturated) (Figure 6). With optimization of the LAMP conditions, the product will reach significant or maximum levels at an earlier endpoint time.

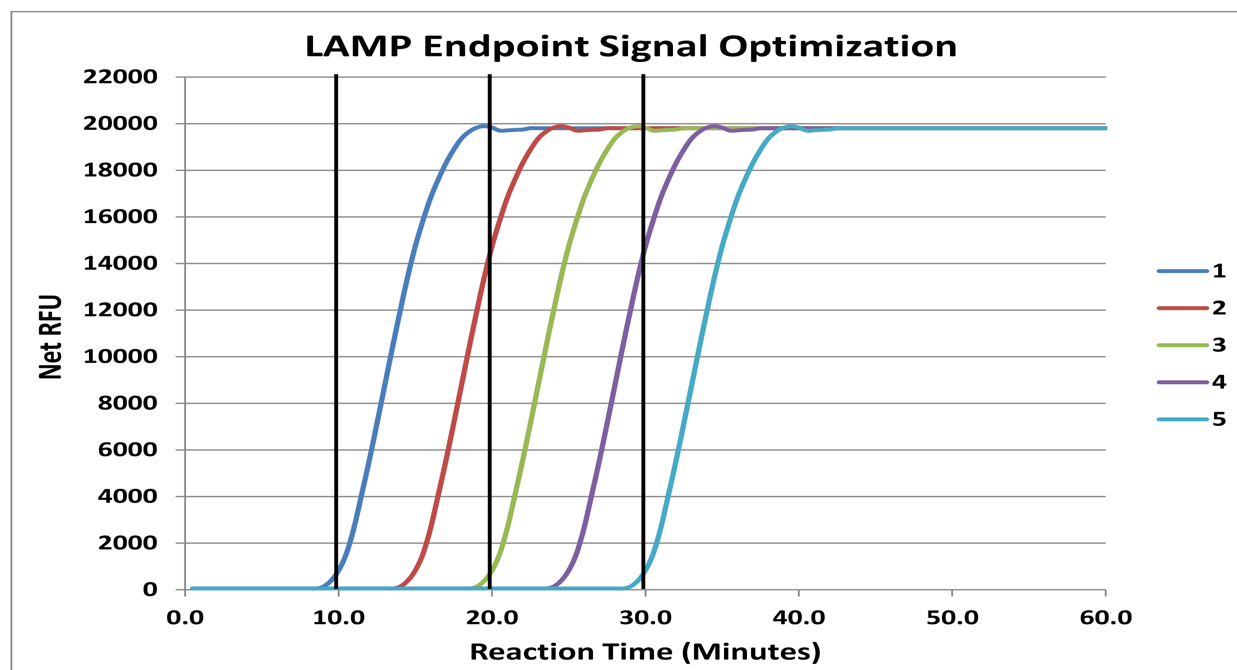


Figure 6. LAMP Optimization by Endpoint Analysis. Theoretical product levels from 5 different LAMP Reactions are shown. Measuring the LAMP product (Net RFU) at the suggested endpoints (vertical black lines) allows detection of positive results. With optimization of the LAMP conditions, the LAMP product will reach maximum levels at earlier endpoint time points

D: Quality Control Assays

Absence of Endonuclease

LavaLAMP™ RNA Master Mix is determined to be free of detectable endonuclease or nicking activity. One µg of supercoiled plasmid DNA is incubated with LavaLAMP™ RNA Master Mix for 16 hours at 70°C. Reactions are analyzed by agarose gel electrophoresis. The LavaLAMP™ RNA Master Mix is deemed to be free of endonuclease or nicking activity if there is no alteration in mobility.

Absence of Exonuclease

LavaLAMP™ RNA Master Mix is tested to be free of contaminating exonuclease activity by incubating 1 µg of Hind III-digested lambda DNA with LavaLAMP™ RNA Master Mix at 70°C for 16 hours. Reactions are analyzed by agarose gel electrophoresis, and the LavaLAMP™ RNA Master Mix is deemed to be free of exonuclease activity if there is no visible alteration in mobility.

Functional Assays

LavaLAMP™ RNA Master Mix Amplification system is tested for performance by isothermal amplification of a target region within the MS2 bacteriophage genome.

E: References and Additional Reading

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 2000 28(12):E63.

Nagamine K, Hase T, Notomi T. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol Cell Probes. 2002 16(3):223-9.

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